Watching Small Molecules Move: Interrogating Ionic Channels Using Neutral Solutes

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Whether they are small enough to wriggle through the current-carrying part of an ionic channel or big enough to be kept outside and thus able to exert an osmotic stress on the channel space, polymers interact with channels in several instructive ways. The osmotic stress of excluded polymers allows one to measure the number of water molecules that come out of the channel in transitions between various "open" to "closed" states. The loss of osmotic activity, due to the partial or completely unrestricted admission of small polymers becomes a measure of the transfer probabilities of polymers from solution to small cavities; it provides an opportunity to study polymer conformation in a perfectly sieved preparation. Current fluctuations due to the partial blockage by a transient polymer are converted into estimates of times of passage and diffusion constants of polymers in channels. These estimates show how a channel whose functional states last for milliseconds is able to average over the interactions with polymers, interactions that last only microseconds. One sees clearly that in this averaging, the macromolecular channel is large enough to react like a macroscopic object to the chemical potentials of the species that modulate its activity.

> "The noise from an open channel can be interesting."
> Charles Pasternak, June 27, 1995

INTRODUCTION

The ability to observe the opening and closing of single ionic channels has become so expectable that the ability to observe can run ahead of the questions we can think to ask. One of the truly remarkable features of single-channel

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measurements is that it is possible to describe many features of single-molecule behavior using classical statistical concepts that are ordinarily applied to large numbers of molecules. It was one of the bolder steps in ionic channel thought when Ehrenstein, Lecar and Nossal in 1970 [1] connected probabilities of conductance states with the conductance-state energies of the single channels they observed in lipid bilayers. To do this they asserted the validity of the Boltzmann relation $p \sim e^{-W/kT}$ to relate the work W needed to be in a given state and the probability p of the channel being in that state. The work W is felt in units of the thermal energy kT that gives the kick to enter the particular state even when the work to get there is much bigger than thermal energy.

Usually when we speak of probabilities of states of macromolecules in solution we think in terms of concentrations or fractions of molecules in a particular state while we simultaneously look at a very large number of molecules that can be in a variety of states. In contrast, with single channels, probabilities are extracted by looking at the one-molecule or few-molecule sample for a long enough time to see its time-average behavior. It's an old hypothesis, a tenet of statistical mechanics but still incompletely proven, that the properties of a very large number of molecules seen at a given moment are the same as the properties of one molecule observed over a sufficiently long time.

And we use this hypothesis to learn about channels.

STRATEGY

Among the many applied variables—voltage, salt concentration, pH, lipid type, etc.—used to control channel opening/closing in reconstituted systems, the osmotic stress of polymers in the bathing solution has begun to allow us to probe the structure of peptide channels. The exposure of channels to neutral solutes such as polyethyleneglycols that are too large to enter some part of the channel lets us guage the size of their aqueous cavities and measure the amount of solute-inaccessible water that flows into and out of the channel when it opens and closes.

If a solute is too big to go into a channel, it is frustrated in its wish to occupy all possible solvent. We might imagine that there is effectively a semi-permeable membrane at either end of the channel (Fig. 1, left) that creates the configuration of a microscopic osmometer.

It is one of the minor miracles of macroscopic thinking applied to small systems that molecular exclusion by this effective microscopic semi-permeable membrane creates an osmotic stress every bit as real as the osmotic pressure detected by a laboratory scale osmometer [2–6]. The excluded large solute acts to draw water to itself, to dry out the inside of the channel, and to close the channel (see Fig. 2).

When a solute is very small compared to the channel, osmotic action is lost. The small molecules swim everywhere at will, not caring whether they are inside a channel or not (Fig. 1, right). They can swim in the same number of water molecules whether the channel is open or closed.
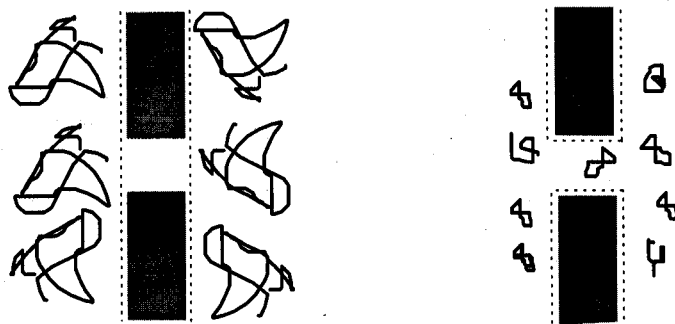
Fig. 1. The ion channel is a filter for "large", sterically excluded polymers but is a passageway for small polymers. Left side: exclusion creates osmotic action on the channel as if there were a semi-permeable membrane (dotted line) across the mouth of the channel. Right side: smaller solutes, able to enter the channel, might still be excluded from a region of the molecular surfaces that compose host membrane and channel structure. In this case there will be relatively slight osmotic action (Figure from reference 2, with permission.)

Between "very small" and "too big" there is a fractional osmotic effect in exact proportion to the extent of exclusion from the internal water space that is lost when the channel closes. A plot of osmotic action on alamethicin channels vs. the size of polyethyleneglycol (PEG) used to stress the open channel (Fig. 3) shows how osmotic action can be modulated by the size of the interrogating polymer. This partial osmotic action is in fact a rather rigorous measure of polymer exclusion [6].

There are in fact other ways to see whether a neutral molecule can get into the channel. These involve looking at the conductance of the channel itself: either its time-average value [8, 9], lowered when there are partially obstructing neutral molecules within, or its variation ("noise") [10], created by the momentary passage of neutral molecules that move through during the relatively much longer open times of the channel.

The clue to the first is to compare the decrease in channel *conductance* with the decrease in solution *conductivity*, which is itself affected by the obstruction of polymer. The decrease in conductivity of the bathing solution is a nearly linear function of PEG concentration [11] (solid line, Fig. 4). It seems not to depend on PEG molecular weight, only on the monomer density of PEG per volume of solution. It is instructive then to see that small-molecular-weight PEG's lower the average channel conductance in approximately the same proportion that they lower bathing solution conductivity (lower dashed line in Fig. 4).

The implication is that average conductance measures the level of PEG population in the open channel even when there is some exclusion; that is, when the slope of channel conductance vs. [PEG] is less than what one would have expected from conductivity vs. [PEG] (Fig. 4). What is very reassuring is that
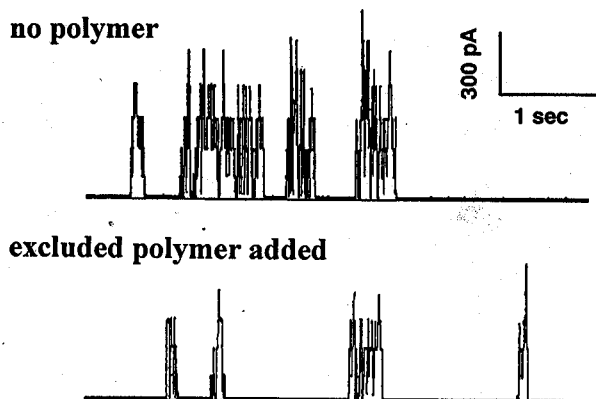
no polymer

300 pA

1 sec

excluded polymer added

Fig. 2. An example of channel closure by the osmotic stress of a "large" polymer. In this case, alamethicin channels are bathed in solutions with and without PEG molecular weight 3400 at a concentration of 15 wt/wt%. The PEG exerts an osmotic pressure which acts to shut the channel. Note the discrete conductance levels of the several states. The probabilities of higher conductance states are therefore lower in the presence of polymer. All other conditions—channels formed in dioleylphosphatidylethanolamine (DOPE) bilayers in 1 M NaCl at 130 mV—are the same in both cases. (Data from ref. 6 with permission.)

the partial entry of polymers inferred this way agrees with that of their partial osmotic action [12].

Channel noise is much more fun to think about. The observation is that in the presence of very large or very small solutes, open channels are not noisy. PEG of intermediate sizes creates lots of open channel noise. Figure 5 shows this for channels in MW 600 PEG bathing solutions, and Fig. 6 plots the variation of noise vs. molecular weight for a range of PEG's at the same solution weight per cent.

The qualitative interpretation is easy. Big guys never get a chance to obstruct ionic flow; small guys go in so easily and in such large numbers that their obstruction gets averaged out to a smooth ionic current vs. time (at an average conductance level that reflects the free entry of molecules equilibrating between channel pore and bathing solution).

Only the mid-size molecules create a significantly irregular flow of ions. The entry of each molecule creates a perceptible drop in current; the probability of entry is low enough that the overwhelming fraction of the time the channel is occupied by either one PEG molecule or none. The ability to measure this current noise is the basis of a "molecular Coulter Counter" [10] allowing estimation of polymer passage times.

Again, the likelihood of polymer penetration inferred from the noise correlates with partial osmotic action [6].
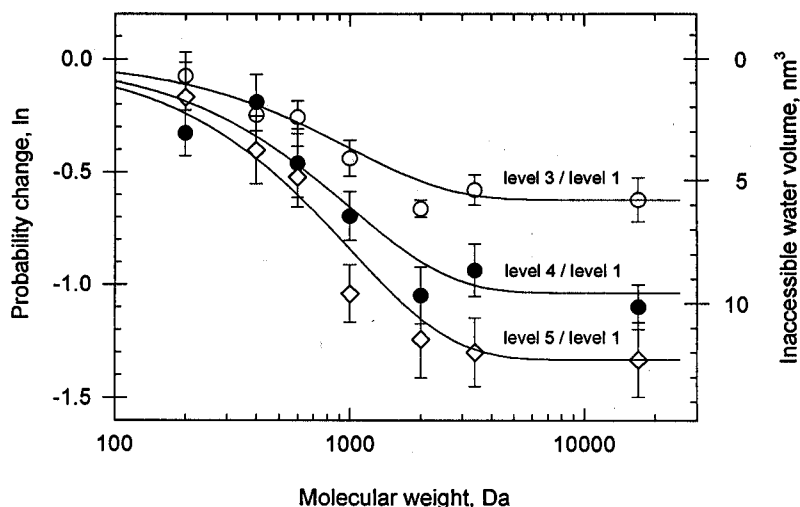
Fig. 3. The progression of osmotic action by polymers of increasing size. The probabilities of higher-conductance states (levels "3", "4", and "5", see Fig. 2 and Fig. 5) of alamethicin relative to the lowest conductance state ("level 1") decrease when the channel is exposed for larger polymers. The osmotic pressure of all solutions was kept at $4.5 \times 10^6 \, erg/cm^3$ for polymers of all molecular weights. All other solution conditions are kept constant [7]. Channels were formed in DOPE bilayers in 1 M NaCl and held at 100 or 110 mV applied voltage. The sensitivity to osmotic stress is in direct proportion to the volume of solute-inaccessible water that is lost by the channel when it goes from level 3, 4, or 5 down to level 1. These volumes are plotted on the right hand oridnate in nm^3 units and reflect the decreasing availability of channel water to polymers as their size increases. (For details see reference 6 from which this picture has been taken and slightly modified.)

INTERPRETATIONS

Now think about points of view.

- To the channel, the polymer is an intruder wanting to dissolve into all available aqueous space—either swimming into the open channel or sitting outside, drawing the water to itself.
- To the polymer, the channel is a barrier posing some energy of entry E making entry difficult to the extent of a biasing factor $e^{-E/kT}$ against occupation of the aqueous space of the channel.
- To the observer, it's a fight between thirsty contestants—and a remarkable chance to watch how solution conditions control the function of a large molecule.

So far, by concentrating on osmotic consequences, we have taken the point of view of the channel. What does one learn from the way the polymer looks at the channel?

To the polymer, the channel is effectively a sieve into which it can enter only in a restricted fraction of the configurations it would ordinarily express in solution. Such exclusion is in fact the basis of using passage of polymers through pores as a way of "sizing" the effective channel opening as, for example, in a
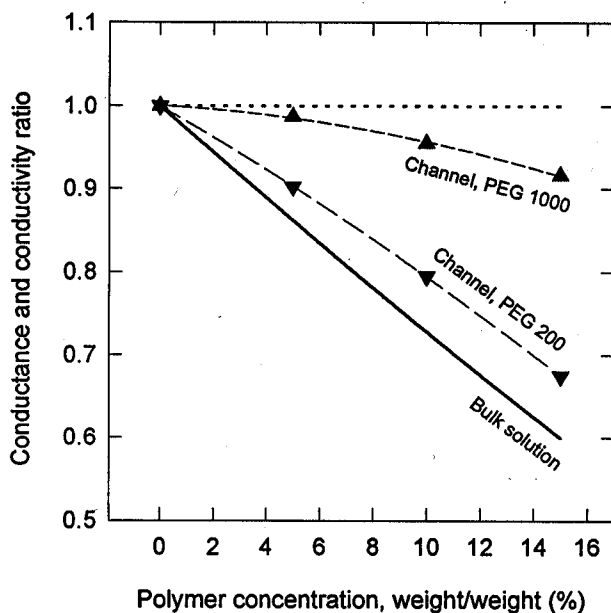
Fig. 4. Solution conductivity (solid line) and channel conductance (dashed lines) are both decreased by PEG in the solution. With all polyethyleneglycols there is a nearly linear relation for *conductivity* and *conductance* vs. concentration of PEG, but the slope is much less for conductance in the presence of large PEG MW1000 (upper dashed line) which cannot enter the channel as easily as PEG MW200 (lower dashed line). Level 2 conductances are used here. Channels were put in diphytanoylphosphatidylcholine (DPhPC) bilayers and bathed in 1 M NaCl solutions at 100 mV applied potential.

recent paper on α-toxin with polyethyleneglycols [13]. The implicit assumption in this sizing is that there should be a cut-off in polymer entry when the radius of gyration of the polymer in solution is comparable to the radius of the channel pore [8]. It turns out that this intuitive idea is actually quite reliable. When we know the channel size independently from conductance, the cut-off of polymer entry measured from partial osmotic action and from noise has a mid-point reassuringly near the expected polymer size (see Fig. 7).

But the cut-off is not completely sharp. Polymers whose molecular weight is twice the nominal cut-off size actually have some probability of entry. They exert less-than-complete osmotic stress. The spread of exclusion vs. polymer molecular weight actually becomes a way to learn about polymer configuration distributions. Between the ability to watch them enter and move through the channel and the fact that natural channels are far more stringently dimensioned than any artificial pores on which such exclusion studies are usually made, we can recognize the possibility to use ionic channels as a new way to study polymer configuration!

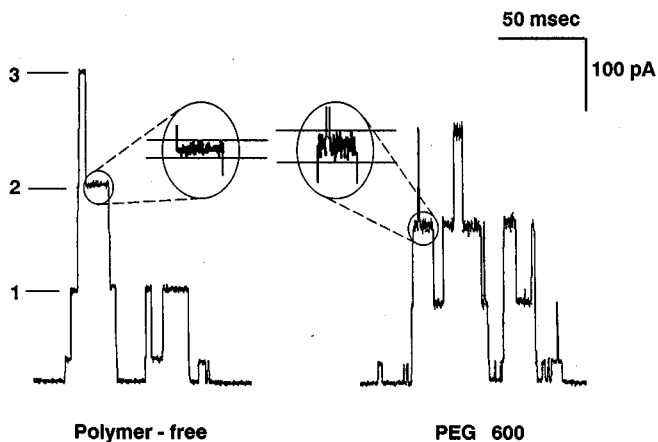To first approximation we can think of the channel selecting only those

Fig. 5. Intermediate-size polymers induce conductance noise in the open alamethicin channel. Current fluctuations in the open state are much larger in the presence of PEG600 (right side) than they are before polymer is added to the bathing solutions. Partly penetrating PEG600 also lowers the average conductance, but this reduction—as fluctuations show—is clearly an average over a range of instantaneous conductances. Alamethicin channels here are formed in DPhPC bilayers bathed in 1 M NaCl solutions at 150 mV. The polymer is added to a concentration of 15 wt/wt%. (Figure from ref. 10 with permission.)

polymers that are in configurations that fit within its walls. If there are $\Omega_{solution}$ polymer configurations in solution but $\Omega_{pore} \lesssim \Omega_{solution}$ configurations within the pore, then configuration selection alone creates an exclusion factor proportional to $\Omega_{pore}/\Omega_{solution}$ [15]. With a larger purpose, we can take the various measures of polymer entry to learn about works of confining polymers under well defined structural and thermodynamic conditions. We will encounter more than questions of configuration, itself not well formulated theoretically for the range of polymer sizes studied. There are other instructive factors such as polymer attraction or repulsion from the channel walls. Moreover, polymer solutions are certainly non-ideal so that partition between bath and channel interior is more involved [6] than the simple ratio of configurations used for illustration here. In fact, measurements of polymer partitioning immediately bring one the opportunity to examine large issues in the field of polymer science completely separate from questions about channels.

And what about the third perspective, that of the observer seeing the conflict between polymer and channel? How does this competition control the response of the functioning state to solution conditions?

BIG/SMALL; LONG/SHORT

Among the many features that make a macromolecule or a molecular assembly "macro" are
(1) the difference in size between it and the species that bathe it
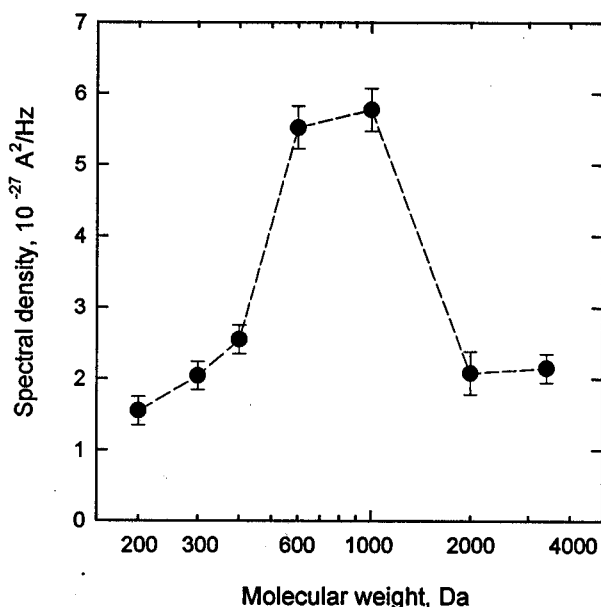and

Fig. 6. The waxing and waning of channel noise as a function of polymer molecular weight. "Medium-sized" 600-to-1000 molecular weight polyethyleneglycols make the most noise. It is also over this size range that the most rapid change in osmotic action occurs in going from "small" to "large" polymers (see Fig. 3). The data shown here are in the form of a power spectral density of open channel current noise (in level 2) averaged over a 200-2000 Hz frequency range; they show a roughly bell-shaped curve as a function of polymer molecular weight. Conditions are as in Fig. 5 (Figure from ref. 10 with permission).

(2) the long times that it stays in a particular state compared to the times of movement of the bathing species.

The difference in molecular size means a difference in the relative number of small vs. large molecules concerned in a macromolecular event. When the alamethicin channel closes by one conductance state, some $\Delta N_w = 110$ water molecules are expelled into the bathing solutions from its polymer-excluding internal space [16]. We think of this ΔN_w as the difference in the number of water molecules associated with the channel peptides in its different conductance states. If we multiply ΔN_w by $\overline{V}_w$, the molecular volume of water, we can speak of the change in the *volume* $\Delta V_w = \Delta N_w \overline{V}_w$ of solute-inaccessible water associated with the channel. The extent of the osmotic action of polymers in the bathing medium working to close the channel is in direct proportion to this volume of water transferred. A change in osmotic pressure $d\Pi_{osm}$ of the bathing solution effects a change in the work of expulsion of this water $\Delta V_w \, d\Pi_{osm}$ [17], but this kind of complete osmotic action is for the case where there is complete exclusion of polymers.

If the added osmotic agent is so small that it can flow freely in and out of the
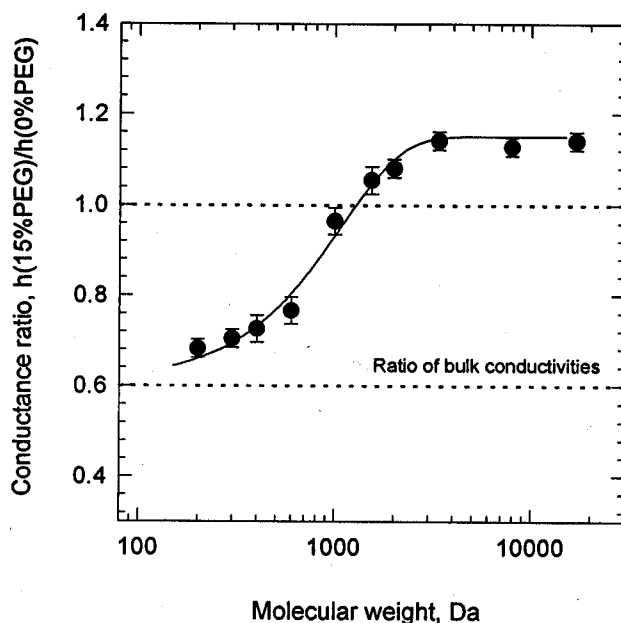
Fig. 7. Different size polymers change alamethicin (level 2) channel conductance as a smooth function of molecular weight. The smaller polymers partition freely inside and outside the channel; their suppression of conductance approaches that of the suppressed conductivity of bulk solutions (also see Fig. 4). Larger polymers not only stay outside the channel but slightly increase the ratio of ions/water inside the channel because of preferential interaction of bathing solution PEG with water. (For details see reference 9). The shape of this curve gives a mid-point for polymer exclusion vs. size near MW 1000 in keeping with noise (Fig. 6) and osmotic (Fig. 3) data. Exclusion vs. PEG size is a similarly smooth function [14]. Channels here are formed in DPhPC bilayers in 1M NaCl solution and are observed at 100 mV applied potential. Data are corrected for access resistance in order to show the conductance of the channel proper (see ref. 9).

open channel, then osmotic action is obviously lost. It is the "filtering power" of the channel that renders it sensitive to excluded agents. If water and polymer both enter an opening channel in exactly the same ratio as that in which the water and polymer cohabit the bathing solution, then there is no osmotic effect of added polymer. Symbolically this says that if ΔN_p is the average number of polymers that go into the opening channel and if $\Delta N_p/\Delta N_w$ is the same as n_p/n_w, the ratio of water to polymer in the bath, then there is no osmotic action. In fact, for all stages of partial exclusion the partial osmotic action is not $\Delta N_w \overline{V_w} d\Pi_{osm}$ but rather $\Delta N_w[1 - (\Delta N_p/\Delta N_w)/(n_p/n_w)]\overline{V_m} d\Pi_{osm}$ (see ref. 6).

Measurements of ΔN_w and ΔN_p show [6, 10] that the *numbers* of polymers and waters can be much greater than the number of channels into which they go.

And the *times* of entry and exit of the diffusing polymers can be very short compared to the times that channels remain open.

If the polymer diffusion constant in the channel is $\sim 10^{-11}$ m^2/sec (see ref. 8), then the time it takes to travel the ~ 5-nm length of a channel is $\sim 25 \times 10^{-7}$ seconds = 2.5 microseconds when we use the form of Einstein diffusion [15]. An open-channel lifetime of ~ 2.5 msec in a particular state is ~ 1000 times the occupation time of a polymer within the channel.

This last point is the occasion of a minor miracle that is at once obvious and frighteningly elusive.

The numbers N_p and N_w are average values of channel occupation over the lifetime of a given state. If the average number of easily exchanging polymers in a channel is two, for example, there will be some fraction of the time that there are three polymers inside; some fraction with one polymer; some with none. If, for partially excluded polymers, the average number of polymers inside the open channel is 1/2, this means there is no polymer within almost half the time, one polymer almost half the time; and two or more inside the channel some small fraction of the time.

Over a several-millisecond duration of channel lifetime, there will be hundreds or thousands of changes in polymer occupation. The channel manages to "compute" a time average over all these occupancy levels and decides to change from "open" to "closed" form on the basis of this time average [16].

What one is observing is the kind of averaging that proteins and other large molecules always have to do to sense the concentration of species that control their activity. In order to respond to those concentrations they must sample the solution for long enough time to "see" how many hits occur per second. This requires that the response of the protein be very slow compared to the rate of hits. An extreme, perhaps trivial, example is proton titration. The pK of a proton-sensing acid is just that pH at which its response is most sensitive to pH; yet it is also the pH at which the site is occupied exactly half the time. Thermodynamically we say that the occupation is 1/2 and know that this rigorously gives the response of the protein. By making the response time of the protein so many orders of magnitude greater than the on/off times of protons, Nature has designed a sensor wherein a single protein molecule can measure their activity and also the activities of other small weakly perturbing solutes [20, 21]. This sensor converts these activities into responses in a time domain created by the large molecule itself, relatively sedate changes far from the frenetic speeds of the small, controlling agents.

With neutral solutes we are seeing something quite remarkable in the mis-match of two kinds of dynamics—the rapid dynamics of diffusion/binding/unbinding vs. the much slower dynamics of channel structure transition. "Microscopic" vs. "mesoscopic" are good words to distinguish these very different times and sizes. During their open time the alamethicin peptides average over ~ 1000 events of polymers passing through them. The free energy, or work of going between long-living open and closed states, is as precise an average as any macroscopic thermodynamic quantity. It turns out that if you do watch one object long enough then indeed its time average equals its ensemble average, but it is apparently a far more complicated matter than just switching back and forth as the polymers roll by.

Think next about the case where polymers are completely excluded and ask what these averages mean. Consider the case of a very high molecular weight PEG in a 15 wt% bathing solution. Fifteen grams of 20,000 molecular weight PEG into 85 grams water means that there are some 6300 molecules of water for each molecule of PEG [22]. The best guess of the number of polymer-inaccessible waters in an open alamethicin channel is 110 waters [6]. This means that a volume of solution in the bath equal to the osmotically responsive volume of the channel will on average contain a polymer only $110/6300 \approx 2\%$ of the time. Most of the time the channel interior would have been devoid of polymer even if there were no effective membrane (Fig. 1) across its mouth. What kind of "complete exclusion" is this anyway? A 2% effect. Too closely regarded, this question brings us dangerously and erroneously near the world of Maxwell Demons and their relatives. But as an example of fundamental statistical mechanics, this complete exclusion is the kind of time average that must hold if the channel is to obey elementary thermodynamic laws. The act of opening a channel contains a work to filter out large polymers; the magnitude of this work is the mean osmotic pressure of the polymer in the solution times the volume of polymer-inaccessible space created by opening.

CONCLUDING THOUGHTS

Recalling the assigned theme of "membrane dynamics", we realize that the combination of noise and osmotic measurements put us in a rather privileged position to watch a molecule decide between different functioning states in response to solution conditions. Channels are at that "mesoscopic" level where we can see macroscopic laws acting on a microscopic system. Biomolecules in general must be large enough and slow enough to be able to distinguish true solution parameters from the very rapid fluctuations in the smaller species that affect transitions between differently functioning states. Noise spectra show the dynamics of the transient polymers; osmotic response shows the averaging over those dynamics to determine the thermodynamic free energies that control conformation.

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7. 4.5×10^6 erg/cm^3 = 4.5×10^5 pascals (J/m^2) = 4.5 bar $\cong$ 4.5 atmospheres.
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11. This has been validated in the concentration range $0 \leq [\text{PEG}] \leq 15$ wt.% (ref. 9).

12. Since polymer osmotic pressure is a function of molecular weight but solution conductivity is independent of molecular weight, there is no easy connection between conductivity and osmotic pressure.

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15. The free energy in this case is purely due to entropy S terms where $S \sim k \ln(\Omega)$ and the free energy or work to place the polymer goes as $G \sim -kT \ln(\Omega)$. The work to transfer the polymer from solution to pore, that is the difference in free energies inside and outside the pores is then

$$W = \Delta G \sim -kT\{\ln(\Omega_{\text{pore}}) - \ln(\Omega_{\text{solution}})\} = -kT \ln(\Omega_{\text{pore}}/\Omega_{\text{solution}}).$$

Exclusion goes as an exponential of the work of transfer $e^{-W/kT} = (\Omega_{\text{pore}}/\Omega_{\text{solution}})$.

16. The data in reference 6 show a large-solute inaccessible volume of $3300\,\text{Å}^3$ coming out of an alamethicin channel when it closes. The number of water molecules coming out is this channel volume divided by the $30\,\text{Å}^3$ volume of a water molecule.

17. See ref. 5 for the discussion of channels and ref 2 for a description of the osmotic stress strategy applied to molecules in solution. For the specific case of alamethicin, please see ref. 6.

18. This diffusion constant is taken from reference 10 to obtain $x^2 \sim Dt$;

$$t \sim x^2/D \sim (5 \times 10^{-9}\,\text{m})^2/(10^{-11}\,\text{m}^2/\text{sec}) = 25 \times 10^{-7}\,\text{sec} = 2.5\,\text{microsec}$$

for a time to pass through the entire channel; for mean occupancy time, divide by 6π.

19. In fact, osmotic response with Boltzmann statistics has been observed for channels that can remain in a given conductance state for ~minutes. See ref. 4.

20. Bezrukov, S. M. and Kasianowicz, J. J. (1993) *Physical Rev. Letts.* **70**:2352–2355.

21. Kasianowicz, J. J. and Bezrukov, S. M. (1995) *Biophysical Journal,* **69**:94–105.

22. Fifteen grams of MW 20,000 PEG contain

$$((0.6 \times 10^{24}\,\text{molecules/mole})/(20,000\,\text{grams/mole})) \times 15\,\text{grams} = 4.5 \times 10^{20}\,\text{molecules};$$

eighty five grams of water (molecular weight 18) contain

$$(0.6 \times 10^{24}/18) \times 85 = 1.83 \times 10^{24}\,\text{molecules}.$$